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(54) Title: NON-FIBROGENIC, ALGINATE-COATED TRANSPLANTS, PROCESS OF MANUFACTURE AND METHOD OF USE THEREOF (57) Abstract A tissue transplant comprises viable, physiologically active, tissue cells and has a non-fibrogenic coating of a divalent metal alginate. The coating has a sufficiently low permeability and a sufficiently large thickness to protect the tissue cells from host immunological agents after transplantation, and is sufficiently permeable and thin to permit the diffusion of sufficient cell nutrients and cell products through the coating required for cell viability. The tissue cells may be pancreatic islet cells, neural cells, renal cortex cells, vascular endothelial cells, thyroid cells, adrenal cells, thymic cells, ovarian cells, hepatic cells, and the like.		

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NON-FIBROGENIC, ALGinate-COATED TRANSPLANTS,
PROCESS OF MANUFACTURE AND METHOD OF USE THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

5 This invention is directed to the field of medical
transplants of cells and tissues, the manufacture of such
transplants, and their use. In particular, this invention
is directed to the coating of transplants with a novel,
highly protective coating of alginate free of transplan-
10 tation-impairing amounts of impurities, the coated tissues
formed by this coating process and the transplants made
using these products.

Discussion of the Background

15 Traditional medical treatments for functional
deficiencies of secretory and other biological organs have
focused on replacing identified normal products of the
deficient organ with natural or synthetic pharmaceutical
compositions. For example, for treating insulin-dependent
20 diabetes mellitus, also known as type I or juvenile onset
diabetes, the normal secretion of insulin by the islets of
Langerhans in the pancreas must be replaced since
functional islets are no longer present in the pancreas.
This pancreatic function is emulated by administering
25 insulin, titrating the injections in response to blood
glucose level measurements. At best, insulin production
and secretion of the islets is imperfectly approximated and
normally is poorly approximated.

30 Organ replacement has also been used. This has
generally required continuous use of immunosuppressive
agents to prevent immunological rejection of the organ,
depriving the patient of the full protective function of
the immune system against disease. This approach has
provided permanent relief only for a limited group of
organs. Attempts to transplant organ tissues into
35 genetically dissimilar hosts without immunosuppression have

been generally defeated by the immune system of the host. Prior to this invention, the application of effective protective barrier coatings to isolate the transplant tissues from the host immune system did not prove to be medically practical for a number of reasons. The coating materials were incompatible with the host system or otherwise unsuitable. The previously developed encapsulation or coating processes did not yield reproducible coatings having the desired porosity and thickness required for the transplanted tissue to have a long and effective functioning life in the host.

To protect transplants from destruction by the immune response of the host animal, various attempts have been made to create a protective barrier between the transplant tissue or cells and the immunological components of the host's system. T.M.S. Chang (Chang, T.M.S., *Science* 146: 524-525 (1964) described the microencapsulation of erythrocyte hemolysate and urease in semi-permeable polyamide membranes. These microcapsules did not survive for long when injected into the blood stream. Mosback et al and Chang et al. described the preparation of semi-permeable microencapsulated microbial cells and viable red blood cells, the latter article mentioning the possibility of using injections of encapsulated cells for organ replacement therapy. (K. Mosbach et al, *Acta Chem.Scand.* 20:2807-2812 (1966); Chang, T.M.S. et al, *Can.J.Psysiol. and Pharmacol.* 44:115-128 (1966)).

Viable tissue and cells have been immobilized in alginate droplets coated with polylysine by Lim et al. (F. Lim et al, *J. Pharm. Sci.*, 70:351-354 (1981)). An attempt to use these coated droplets to correct the diabetic state of diabetic animals was reported by Lim et al, (Lim et al, *Science* 210:908-909 (1981)). U.S. Patent Nos. 4,251,387, 4,324,683, 4,352,883, 4,407,957, 4,663,286, and 4,803,168 relate to this research. These products, however, have not been successful for the long term correction of the diabetic state of animals, and they have not proven

suitable for transplanting tissues such as pancreatic islets in humans.

Substantial additional efforts made by Goosen et al. to develop transplants encapsulated in calcium alginate droplets reacted with polylysine were also unsuccessful in providing protected transplants suitable for transplantation. (U.S. Patent Nos. 4,673,566, 4,689,293, 4,789,550, 4,806,355, 4,789,550, for example).

Lim et al. reported the prolonged reversal of the diabetic state of NOD mice with xenografts of microencapsulated rat islets, using alginate-polylysine capsules. (Lim et al., Diabetes 40:1511-1516 (19)).

U.S. Patent 4,744,933 describes encapsulating solutions containing biologically active materials in an outer membrane of inter-reacted alginate and polyamino acid.

U.S. Patent 4,696,286 describes a method for coating transplants suitable for transplantation into genetically dissimilar individuals by coating the transplant with a surface-conforming bonding bridge of a multifunctional material that binds chemically to a surface component of the transplant followed by a semipermeable, biologically compatible layer of a polymer that binds chemically to the bonding bridge layer.

Hackel et al and Kerstan et al report the use of calcium alginates for immobilization of microbial cells and enzymes. (Hackel et al., *J. Appl. Microbiol.* 1:291-296 (1975); Kerstan, M. et al, *Biotech. and Bioeng.*, 19:387-397 (1977)). Nigam et al. and publications cited therein describe methods for coating living cells in an outer membrane of calcium alginate by dropping a cell-containing calcium solution into an alginate solution and further incubating the capsules in a calcium solution. (Nigam et al, *Biotech. Tech.*, 2:271-276 (1988))

Plunkett et al. describe an angiogenesis model using tumor cells entrapped in alginate beads. (Plunkett et al, *Lab. Invest.*, 90: 6204-6205 (1990)). A spray of sodium alginate-cell solution droplets was contacted with aqueous calcium chloride solution to form calcium alginate beads.

Pump speed and air pressure were used to control the droplet size in the spraying process.

5 Calcium alginate coated implants, however, have not been previously considered suitable for use in transplanting tissues because the coated transplants did not survive in the host systems. Alginates in the form obtained from seaweeds are mixed polymers of guluronate and mannuronate containing various levels of other materials.

10 The gelation of calcium alginate is primarily caused by calcium ion bonding with the guluronic acid moieties of high guluronate polymers, which have higher porosity and provide higher levels of cross-linkage resulting in a strong protective barrier for the transplants. Skjak-Braek, alternatively, discloses the use of mannuronan C-5
15 epimerase, an enzyme capable of converting D-mannuronic acid residues which are already present on the alginate polymer into L-guluronic acid. (G. Skjak-Braek, *Biochem. Soc. Trans.*: 20-26 (1992)).

20 Although alginates have been applied to coating food and pharmaceutical products, the use of alginates for coating living cells to produce a non-immunogenic coating has heretofore not been achieved. Those working in this field have recognized that it is necessary to purify alginates for cell coating applications. For example,
25 filtration has been suggested as a means to remove proteins and polyphenols. However, even the purified alginates themselves are immunogenic. Soon-Shiong et al. have observed fibrous overgrowth of implanted alginate microcapsules in large animals. (Soon-Shiong et al, *Trans. Proc.*, 23:758-9, 1991)). This study found that commercial
30 alginates are often contaminated with polyphenols and other immunogenic materials. However, even when purified, commercial alginates having high mannuronic acid content remained immunogenic and capable of activating macrophages
35 in vivo and producing fibrotic overgrowth. Similarly, Espevik et al. suggest that all alginates are inherently fibrogenic. (Espevik et al, *Cell Trans.* 1:165 (1992)). T. Zekorn, *Cell Trans.* 1:176 (1992)). Others have reported

that high mannuronate alginates stimulate human monocytes to produce cytokines (M. Otterlei, et al., *J. Immunother.* 10:286-291 (1991)), or enhance macrophage migration (M. Fujihara, and T. Nagumo, *Carbohydrate Research* 243:211-216 (1993)), suggesting why they are not biocompatible. Thus, it appears to be the consensus in the prior art that alginate, at least alginate with a significant fraction of mannuronate, stimulates cytokine production, macrophage migration, and is inherently fibrogenic. Despite recognition in this field that purified alginates might be used for coating cells, long term non-immunogenic coatings have up to this time not been achieved.

Compositions of alginates and methods for purifying and fractionating alginates have been generally described (Haug, A., *Composition and Properties of Alginates: Report No. 30*, Norsk Institutt for Tang-og Tareforskning (Norwegian Institute of Seaweed Research) (1964); Haug, A. *Acta Chem. Scand.* 13:601-603 (1959); Haug et al, *Acta Chem. Scand.* 19:1221-1226 (1965); Haug et al, *Acta Chem. Scand.* 21:691-704 (1967); Smidrød et al, *Acta Chem. Scand.* 22:1989-1997 (1968); and Skjåak-Bræk et al, *Biotech. and Bioeng.* 33:90-94 (1989)). Correlations between the chemical and physical properties of alginate gel beads have also been reported (Martinsen et al, *Biotech. and Eng.* 33:70-89 (1989)).

Alginate coatings having a thickness greater than 200 μm have been reported to lack the permeability required for permitting the flow of nutrients and cell products through the coating in amounts sufficient for long term viability of the coated transplant while implanted in the host system. (Chicheportiche et al, *Horm. Met. Res. Suppl.* 26:209-213 (1990)).

A need, therefore, continues to exist for novel coated transplants which are substantially not rejected by the host immune system.

SUMMARY OF THE INVENTION

This invention relates to coated viable, physiologically active, tissue or cells for transplantation which are physiologically acceptable to the host, and which effectively provide prolonged protection of the transplanted tissue cells from destruction by the host immune system.

This invention also relates to tissue cells enclosed in a coating capsule having a thickness permitting the diffusion to the transplanted cells or tissue of the amounts of nutrients and other substances required for their health, long life and effective function after transplantation, and a permeability allowing the effective diffusion and release of transplanted tissue or cell products into the host system.

This invention further relates to an effective transplantation tissue coating material which is physiologically acceptable, non-fibrogenic and non-toxic to the host, and which can be used to provide a coating having the characteristics described above.

Still part of this invention is a manufacturing process for effectively coating transplant tissues and other biological substances with a complete barrier coating which is physiologically acceptable, non-fibrogenic and non-toxic to the host and which provides a complete barrier coating with a controlled thickness and permeability to intermediate size proteins.

The coating is substantially free from fibrogenic concentrations of fucose, polyphenol and protein. The amount of fucose groups in the coating is, in general, less than about 0.2 micrograms per milligram of sodium alginate (even less than about 0.02 wt%) and the amount of polyphenol groups is, in general, less than about 2.0 micrograms of tannin-equivalents per milligram of sodium alginate (even less than about 0.2 wt%).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

This invention arose from a desire by the inventors to improve on prior art coatings for tissue transplants, and to provide a composition which, upon being coated onto a tissue to provide a tissue transplant, substantially fails to evoke detrimental reactions upon implantation into a host, such as fibrogenesis and/or host rejection.

The present inventors have discovered that the drawbacks of prior art transplants were the result of several factors, such as certain natural materials present in commercially available alginate preparations being fibrogenic to the host tissue surrounding a transplant. This fibrogenicity, in turn, leads to the encapsulation of the transplant in an impervious, poorly vascularized layer of scar tissue which causes transplant necrosis and disfunction. The present inventors also found that the prior art had failed to remove from the alignates sufficient amounts of substances containing fucose, polyphenols and protein which are instrumental in the developing fibrosis. Although prior to the present invention, transplanted pancreatic cells were said to produce insuline, inspection of the transplanted coated cells always revealed the presence of significant fibrosis. On the contrary, transplants of cells coated with the purified alginates of this invention substantially fail to produce fibrosis.

The present inventors have developed and provide herein efficient procedures for the mass production of transplants having alginate coatings with a thickness of less than about 200 μm , and have found that they can be used upon transplantation to restore, e.g., pancreatic islet function indefinitely.

Prior to this invention, reacting the outer surface of alginate coatings with polylysine was reported to be necessary for alginate coated transplants. The present inventors have developed fully operable, non-fibrinogenic coatings with perfected permeabilities which do not require a secondary reaction of the outer coating with polylysine.

One aspect of this invention, thus, is a novel, alginate composition which is substantially non-fibrogenic. This composition is provided substantially free from substances which may impair the acceptance by the host of
5 transplants coated with divalent metal ion alginate gel products thereof.

In the present invention, an initial fibrogenic alginate preparation is purified to yield a non-fibrogenic alginate suitable for coating cells with a non-fibrogenic
10 coating. Suitable initial alginate preparations are preferably obtained by isolating alginates from brown algae and are commercially available. In this invention, the initial alginate preparation is contacted with a divalent metal ion-chelating agent to remove divalent metal ions,
15 and then contacted with high surface area, bleached, activated carbon. The carbon adsorbs polyphenols together with associated protein and fucose moieties. Compositions containing protein, polyphenols, and fucose moieties are collectively referred to in the art as "fucans". After
20 treatment with carbon, the alginate is precipitated from solution, washed and then filtered to remove additional impurities and to provide the non-fibrogenic alginate of the present invention.

The term "non-fibrogenic" as used herein means a
25 composition which, when used to produce an implant having a diameter of 500 microns or less, does not induce bioisolation and resulting tissue death of the implant by the immune system of a host through the process of fibrosis and macrophage overgrowth for at least 60 days, preferably
30 at least 6 months, more preferably at least 12 months, and most preferably at least 18 months after interperitoneal injection of an alginate coated implant into a host BALB/c mouse or mongrel dog. Bio-isolation and tissue death can be determined, for example, by monitoring insulin
35 production from a pancreatic islet tissue implant or by monitoring the maintenance of euglycemic in a diabetic host. The non-fibrogenic alginate of the invention is

suitable for coating tissues or single cells using known coating technology.

5 The coated transplant tissues and cells of this invention are effective for implantation into a host animal by simple injection through a hypodermic needle having a
10 needle diameter sufficient to permit passage of a suspension of coated cells therethrough without damaging the tissue coating. For implantation, the coated transplant tissues are formulated as pharmaceutical
15 compositions together with a pharmaceutically acceptable carrier. Such compositions should contain the highest number of coated transplant capsules which can be effectively injected into a mammal in order to provide a sufficient number of transplant capsules during
transplantation.

The term "transplant", as used herein, is defined to include all living tissues, cells, and biologically active substances intended to be implanted into the body of a host animal and the act of implanting these tissues and cells.
20 These tissues and cells include, without limitation, tissue and cells removed from a donor animal, tissue and cells obtained by incubation or cultivation of donor tissues and cells, cells obtained from viable cell lines, biologically active products of cells and tissues, and the like. Any
25 type of tissue or cells for which transplantation is desired can be coated and transplanted according to this invention. The most important tissues for transplants are secretory organ tissues, where transplantation from a donor organ to a host animal is desired to at least partially
30 replicate the donor organ's action in the host system. Preferred donor tissues are pancreatic islets, hepatic cells, neural cells, renal cortex cells, vascular endothelial cells, thyroid cells, adrenal cells, thymic cells and ovarian cells. However, other types of cells may
35 also be utilized.

The process of this invention is described hereinafter for the preparation and transplantation of pancreatic islets and islet cells by way of example, for purposes of

clarity of explanation and not by way of limitation. This process can be equally well applied to other organ tissues as will be readily apparent to a person skilled in the art, with conventional and obvious modifications as required to
5 accommodate any uniquely different requirements of the different tissues. Applications of the process to all tissues and cells suitable for transplantation are intended to be within the scope of this invention.

Isolated pancreatic islets (or other cells or tissues
10 suitable for transplantation) may be prepared by conventional procedures to separate them from extraneous tissue and donor substances.

To prepare the non-fibrogenic alginate of the invention, an initial alginate preparation is dissolved in
15 water or buffer, preferably containing a divalent metal ion-chelating compound such ethylenediamine tetraacetic acid (EDTA), among others, and then contacted with a high surface area, activated carbon to remove any fucans and other contaminants present by adsorption. About fifty
20 grams of alginate is usually dissolved in about 1 to 10, and more preferably about 3 to 8 liters, of water. Suitable activated carbon includes any high surface area activated carbon having a particle size of about 100 mesh or finer. Preferably, very fine activated carbon powder
25 having a surface area of at least about 1,000, and preferably at least about 1,500 m²/g, may be used. Suitable activated carbon is commercially available.

The activated carbon is preferably bleached to oxidize and remove organic contaminants prior to use. The
30 activated carbon may be bleached using a known bleaching agent such as sodium hypochlorite, and the like. The carbon may be bleached by stirring it with a dilute solution of the bleach for a time sufficient to remove any contaminants from the surface of the carbon. Generally,
35 stirring the activated carbon with an about 0.005 to 0.50 M, and more preferably an about 0.08 to 0.10 M, sodium hypochlorite solution for about 5 to 30 minutes, and preferably about 10 to 20 minutes, which is is sufficient

to oxidize the activated carbon. After oxidation, the activated carbon may be removed from the dilute bleach solution by centrifugation or filtration, washed with water or ethanol, and dried. The ratio (w/w) of initial alginate to activated carbon is usually about 1:1 to 1:20, and more preferably about 1:2 to 1:8. Obviously, the amount of activated carbon may be adjusted as necessary to insure the removal of sufficient contaminants to achieve the minimum amounts permitted by this invention.

The alginate solution and bleached carbon may be contacted by simple mixing, shaking or rolling, and the bleached carbon may then be removed by conventional centrifugation and filtration. Preferably, the filtration is conducted using sequentially finer sub-micron filters.

A dilute monovalent cation salt solution is then added to the filtered alginate solution in an amount sufficient to exchange metal ion binding sites on the alginate. Any soluble monovalent cation salt may be used. Sodium or potassium chloride solutions of about 0.01 to 1.0 M are, however, preferred.

The alginate may then be precipitated from the resulting solution by addition of ethanol with stirring. Generally, the ratio (v/v) of ethanol to alginate solution is about 0.25 to 2.0, and preferably about 0.5 to 1.5. The precipitated alginate may then be recovered by filtration, washed with ethanol and dried to remove traces of ethanol.

The alginate obtained as described above is directly suitable for coating a transplant tissue or cells without the use of additional coating compounds such as homopoly(amino acids), e.g. polylysine or polyaspartic acid. However, it is sometimes desirable to chemically modify the properties of the alginate to tailor the alginate coating to specific uses. Important properties of the alginate coating materials include, for example, a well defined and controlled pore size, coating thickness, viscosity of coating solutions, mechanical strength, etc.

The average molecular weight and overall mannuronate to guluronate molar ratios are initially substantially

determined by the origin of the material, but may be further adjusted by physical and chemical methods, as well. The molecular weights may be reduced, for example, by partial acid hydrolysis, thermal degradation or sonication. High molecular weights may be obtained by controlled precipitation methods with the concomitant alteration of the alginate composition, or by dialysis, molecular filtration, or gel exclusion chromatography. The mannuronate to guluronate ratio and sequence distribution may be increased or decreased by selective precipitation or solubilization by mono- and di-valent metal cations, organic solvents or acids. Adjustment of these characteristics provide optimum results with different tissue transplants.

The concentration of an alginate in solution is a function of the physical properties of the alginate. At very low concentrations, the coating morphology is poor resulting in ineffective coatings. At very high concentrations, the viscosity is too high to form good coatings. Preferably, the molecular weight (kilodaltons) of the alginate is in the range of about 2 to 300, more preferably about 4 to 250, and even more preferably about 6 to 120. If the initial molecular weights are high, they may be reduced by mild acid hydrolysis of the alginate. The initial or purified alginate may be dissolved in a dilute acid solution and gently heated until the desired molecular weight is obtained. Dilute solutions of mineral acids such as HCl, etc., having a concentration of about 0.1 to 0.5 M may be used. The degree of hydrolysis may be controlled by monitoring the molecular weight of the alginate and neutralizing the hydrolysis reaction when the desired molecular weight is obtained. Obviously, higher acid concentrations result in faster hydrolysis. Alternatively, a few initial test reactions are usually sufficient to determine appropriate hydrolysis conditions.

The porosity and mechanical strength of the coating are also a function of the relative amounts of mannuronate (M) and guluronate (G) in the alginate polymers. Preferably,

the amount of M, calculated as $M/(M+G)$, in the alginate is in the range of about 0.1 to 0.95, more preferably about 0.15 to 0.85, and even more preferably about 0.25 to 0.75. The relative amount of M and G in the alginate may be
5 adjusted by dissolving precipitated alginate in a dilute (e.g., about 0.05 to 0.50 M) potassium chloride solution to redissolve G-rich fractions while M-rich fractions are left in the precipitate. The insoluble material may be collected by centrifugation. The redissolved G-rich
10 material may then be reprecipitated by addition of ethanol. By repeating this process, any desired relative proportion of M and G in the alginate may be obtained.

Homopolymeric alginate sequences (polymannuronate and polyguluronate) are generally acid-insoluble, whereas
15 alternating mannuronate-guluronate sequences are for the most part acid-soluble. By extracting the alginate with an acid solution of pH about 1.5 to 2.5, and preferably pH about 2.0, it is possible to selectively solubilize homopolymeric-rich alginates. Additionally, M-rich
20 alginates are preferentially solubilized relative to G-rich alginates. The treatment of an alginate with an acidic solution, therefore, precipitates G-rich alginates preferentially leaving M-rich alginates in solution. The separation of the precipitate from the solution thus
25 provides both G-rich and M-rich alginate fractions. The G-rich alginates present in the solution may be precipitated by addition of calcium ions or ethanol. Alternatively, the G-rich alginates may be obtained by precipitation of the G-rich fractions with calcium ions while leaving the M-rich
30 fractions in solution. After separation of the precipitate from the solution, the M-rich alginate fraction may be precipitated from solution by addition of acid or ethanol. The proportions of acid and/or calcium precipitated materials may be controlled by adjusting the pH and the
35 calcium concentration, respectively.

It is also possible to obtain specific relative amounts of M and G in the alginate coating by mixing different M-rich fractions and G-rich fractions obtained as described

above. By sequentially adding small portions of G-rich material to M-rich material, the amount of G in the overall alginate composition may be gradually increased, thereby increasing the number of divalent metal ion binding sites in the overall coating, increasing the structural rigidity of the coating, and producing larger pore sizes. For any particular mixture of M-rich and G-rich fractions, the relative amount of M or G in the alginate may be readily determined by NMR spectroscopy. ¹³C-NMR spectroscopy is a rapid and inexpensive method for determining the M/G ratio and disaccharide sequence frequencies but ¹H-NMR may also be used to determine M/G ratios. The average molecular weight and porosity of the alginate coating may be adjusted by mixing M-rich and G-rich fractions in different proportions.

The alginates of the present invention are non-fibrogenic, having a hydrolyzable fucose sugar content of about 0.2 or less micrograms per milligram of sodium alginate (even about 0.02 wt% or less), preferably less than about 0.1 micrograms per milligram sodium alginate (even about 0.01 wt.% or less), and even more preferably less than about 0.05 micrograms per milligram sodium alginate (even about 0.005 wt.% or less). The fucose sugar levels in the alginates may be determined by conventional neutral sugar analysis, such as is described in Example 4 below.

The alginates of the present invention contain a non-fibrogenic amount of polyphenols, such as tannins or phloroglucinol. To determine the level of polyphenols in the alginate of the present invention, the inventors developed a novel assay based on a standard method for the measurement of tannin levels in water. (See, *Hach's Water Analysis Handbook*, Second Edition (1992), Method 8193, adapted from M.B. Kloster, *Journal of the American Water Works Association* 66:44 (1974)). Tannins are polyphenols and the novel alginate assay of this invention for polyphenols gives the polyphenol content in terms of tannic

acid or "tannin-equivalents" based upon standard tannic acid solutions of known concentration.

5 In the present assay for polyphenols in alginates, the alginate samples are prepared at a concentration of about 1 wt.% in water. An aliquot of the alginate sample is placed into a test tube and an equal volume of water is placed into a control test tube. Similar volumes of a sodium carbonate solution and of TANNIVER® 3 reagent (sodium tungstate, phosphoric acid, hydrochloric acid, 10 anhydrous sodium molybdate, lithium sulfate, and water plus 1% of other reagents) are added to both the sample the and control, the solutions are then mixed and incubated at room temperature for about 30 minutes. The absorbance of the sample and control is then measured at about 700 nm.

15 A suitable standard curve for tannic acid may be prepared using tannic acid standard concentrations varying over a range of about 0.1 to 10 µg/ml. After correcting the absorbance of any sample by subtracting the absorbance of the control, the absorbance of the alginate sample may be plotted against a standard curve prepared from the 20 standard tannic acid solutions to determine the number of micrograms of tannic acid present per milligram of alginate sample or the micrograms of "tannin-equivalents" per milligram of alginate. When tested with the polyphenol/tannin assay described above, the alginates of 25 the present invention are shown to contain about 2.0 micrograms or less of tannin-equivalents per milligram of sodium alginate (even about 0.2 wt.% or less). Preferably, the alginates contain about 1.0 micrograms of tannin-equivalents or less (even about 0.1 wt.% or less), and more 30 preferably about 0.75 micrograms of tannin equivalents or less (even about 0.075 wt.% or less) per milligram of sodium alginate.

35 The method of the present invention allows the preparation of an alginate gel coating suitable as a protective barrier which excludes immunologically effective concentrations of agents of the host immune system from contracting the tissue and having the permeability required

to permit sufficient diffusion of nutrients and other substances so that they become in contact with the transplants required for their long life and viability.

5 The viscosity of coating solutions of the alginates having a concentration of about 0.7 to 2.5 weight percent alginate should have a viscosity of about 10 to 250 centipoises and preferably about 20 to 100 centipoises, at 25°C.

10 In the first step of the process of this invention, isolated pancreatic islets (or other cells or tissue) may be washed with isotonic saline and suspended in a solution of purified alginate. Optionally, but not necessarily, the washed cells may be pretreated with an aqueous solution of poly-L-lysine to increase the bonding of the cells with the
15 alginate, and then rinsed with saline.

The cell suspension in the alginate is formed into droplets, and the droplets are contacted with a divalent metal, preferably an alkaline earth metal, salt solution to gel the alginate. The droplets may be formed by any
20 conventional procedure. For example, alginate droplets may be formed by emulsifying a solution of sodium alginate containing cellular material to form droplets of sodium alginate and cells, and gelling the droplets with calcium chloride (U.S. Patent 4,352,883). Alginate droplets may
25 also be formed with a syringe and pump to force the droplets from a needle, using a laminar flow air knife to separate the droplets from the tip, the droplets being gelled by collection in a calcium chloride solution (U.S. Patent 4,407,957). Alginate droplets may also be formed by
30 expulsion from a hypodermic needle, and then allowing the droplets to fall into a calcium chloride solution (Nigam et al, *Biotechnology Techniques* 2:271-276 (1988)). In addition, droplets may also be injected into a concurrently flowing stream containing calcium chloride (U.S. Patent
35 3,962,383). Spraying alginate solutions through a spray nozzle to form a mist of droplets which was collected in a calcium chloride solution may also be utilized (Plunkett et al, *Laboratory Investigation*. 62:510-517 (1990)).

Alginate droplets may also be formed using a combination of a needle and a pulsed electrical electrostatic voltage to form uniform droplets (U.S. Patent 4,789,550 to Hommell et al.). The alginate solution may be forced through a needle tip to form a droplet, and the droplet pulled from the needle by a changing electrostatic field between the needle tip and a calcium chloride solution placed below the needle tip. The droplet receives a charge of one polarity from the needle, which is opposite the charge in the calcium chloride solution. When the voltage difference between the droplet and the oppositely charged calcium chloride solution reaches a threshold value at which the attraction by the solution on the droplet exceeds the force of interfacial tension holding the droplet on the needle tip, the droplet is pulled free to fall into the calcium chloride solution. The electrostatic field may be fluctuated using a square wave form to create a succession of voltages crossing the threshold value, thus producing a continuous series of droplets, one per square wave cycle. This process does not appear to provide the small droplets and thin coatings required for effective transplantation.

A preferred drop forming and gelling procedure for use herein is described in U.S. patent application Serial No. 07/890,982, filed May 29, 1992, the entire specification and drawings of which application, and more particularly the sections describing the drop forming and gelling procedure, being incorporated by reference herein for a more complete description of this process.

The tissue containing viable, physiologically active, tissue cells such as pancreatic islet cells is provided a coating having a thickness of about 10 to 200 μm . The coating comprises a divalent metal alginate gel, preferably a calcium alginate, magnesium alginate, or mixtures thereof, the metal alginate having been formed from an alginate free from impurities which would impair the viability and long life of tissue transplants coated with the alginate. The coating capsule has preferably a

sufficiently low permeability and a sufficiently large thickness to protect the tissue cells from host immunological agents after transplantation, the coating also being sufficiently permeable and thin to permit the diffusion of sufficient cell nutrients and cell products through the coating required for cell viability. The coating preferably has a thickness of at least about 10 μm and less than about 50 μm . Most preferably, a single cells or 1-2 cell(s) are present within a single capsule. If desired, a plurality of alginate coatings may be applied over the cells to produce a multilayered capsule.

If a further reduction of molecular permeability is desired or a reduction of swelling of the alginate coating is required, the alginate gel coating may optionally, but not necessarily, be cross-linked with poly-L-lysine or other physiologically acceptable, non-toxic polycation polymer by immersing the coated tissue in a solution of the polycation polymer. The reduction in permeability is a function of the degree of polymerization of the polycation, the reaction of the polycation with the alginate coating, the concentration of the polycation in solution, and the incubation time. The selection of the optimum polycation and the reaction conditions is conventional and fully within the skill of the art. The reaction may be terminated by depletion of the polymer from the solution or by dilution of washing.

Polylysine, and polycations in general, induce fibrosis and a further treatment of the alginate-polycation complex is desirable to improve biocompatibility of the coated product. The immersion of the polycation reacted products in a solution of sodium alginate to react free epsilon-amino groups at the coating surface leads to an ion-exchange reaction in which the metal alginate core is partially or completely solubilized by depletion of the divalent metal cations therefrom. However, if trace amounts of soluble alginate remain after such treatment, a slow diffusion of the liquified materials from the capsule may lead to a fibrotic reaction, particularly when the

alginate is known to induce fibrosis in its soluble form. If the alginate treated products are treated with a divalent metal ion before complete solubilization of the alginates and their removal from the core, the metal ion
5 may react with the sodium alginate migrating outwardly across the coating layer, thereby compromising the distinctness of the membrane and increasing the likelihood of fibroblastic adhesion to the coating surface.

Therefore, if alginate is to be used in the outer
10 coating, the reaction should be carried out to completely dissolve the core gel either by ion-exchange or chelation of the calcium ion, for example with sodium citrate or EDTA. The product may then be washed exhaustively with several changes of wash medium or by percolation, allowing
15 ample time for the soluble alginate to diffuse entirely out of the coating. As the alginate diffuses outwardly through the coating, free residual amino groups of the polycation may react therewith.

Preferably, a negative charge is applied to the
20 polycation-complexed, alginate-coated tissue transplants by reacting them with polyaspartic acid. Because of its lower binding affinity, polyaspartic acid is less likely to complex and deplete metal ions from the primary alginate gel coating. It reacts with the polycation without
25 dissolving the primary alginate coating. The use of polyaspartic acid as the final reactant provides several advantages over the use of an alginate final complex. It provides greater mechanical strength, smaller coated product diameter, and low permeability due to the
30 additional cross-linking and volume reduction of the condensed coating.

This invention is further illustrated by the following specific but non-limiting examples. Percents are given in weight percents and temperature in degrees Centigrade,
35 unless otherwise specified. In these examples, procedures which have been reduced to practice in the laboratory are presented in the past tense, and procedures which are

constructively reduced to practice in this application are presented in the present tense.

EXAMPLES

Example 1-High Mannuronate Alginate Preparation

5 50 g low viscosity sodium alginate (LV Alginat, KELCO Div. of Merck & Co.) isolated from *Macrocystis pyrifera* were dissolved in 5 liters of water and filtered through a 50 micron mesh to remove particulates. 18.6 g disodium EDTA were added to the solution and dissolved. The
10 solution was mixed on a roller mill with 200 g hypochlorite-bleached activated carbon (Mallinckrodt activated carbon powder) for 30 minutes to remove organic contaminants such as polyphenols and fucose sugar residues. Activated carbon was then removed by centrifugation for 30
15 minutes. The resulting solution was sequentially filtered through filter paper, a 0.45 micron filter, a 0.22 micron filter and a 0.1 micron filter. 30 g. sodium chloride were then added to the filtered solution and dissolved by rolling on a roller mill. The alginat was precipitated
20 from solution by the addition of 5 l neat ethanol. The sample was centrifuged for 30 minutes to obtain an alginate pellet and the alginate pellet was suspended in ethanol and then teased apart with tweezers to insure complete washing of the sample. Excess ethanol was removed by squeezing and
25 pressing the precipitate. The resulting precipitate was dried in an oven, under vacuum, at 60°C.

Example 2-High Guluronate Alginate Preparation

 80 g protan alginate were dissolved in 89 l water by rolling on a roller mill. The solution was filtered
30 through a 50 micron mesh to remove particles, and then mixed on a roller mill with 320 g of bleached, activated carbon with continued mixing for 30 minutes. The activated carbon was then removed by centrifugation for 30 minutes. The resulting solution was sequentially filtered through
35 filter paper, a 0.45 micron filter, a 0.22 micron filter and a 0.1 micron filter. 163 g magnesium chloride were

then added to the solution and dissolved by rolling on a roller mill. 210 ml of a 1.7% calcium chloride solution were then added and mixed by rolling on a roller mill for 30 minutes. The resulting solution was centrifuged for 30 minutes to produce an alginate pellet. The alginate pellet was dissolved in 3.0 liters of 0.1M EDTA, pH 7.0 by rolling on a roller mill. The pH of the solution was adjusted to pH 7.0, as needed. 20 g sodium chloride were then added to this solution and dissolved.

Alginate was precipitated from the solution by the addition of 5 l of neat ethanol, followed by centrifugation for 30 minutes to obtain an alginate pellet. The alginate pellet was then suspended in ethanol and tweezed apart with tweezers to insure complete washing of the sample. Excess ethanol was then removed by squeezing and pressing the precipitate. The alginate precipitate was then dried in an oven, under vacuum, at 60°C.

Example 3-Fucose and Tannin Equivalent Content Determination

Standard tannic acid solutions were prepared by diluting a freshly prepared tannic acid solution (0.1 mg/ml) to obtain standard solutions having a concentration of 10.0, 5.0, 3.0, 1.0, 0.4 and 0.1 µg/ml. A tannic acid standard curve was then prepared by plotting the concentration of tannic acid against the absorbance of each sample read at 700 nm in a spectrophotometer.

Sample solutions of the alginates of Examples 1 and 2 were prepared at 1 wt.%. A 2 ml aliquot of each sample was placed into a 5 ml test tube. Two ml of water were placed into a test tube as a control. Sodium carbonate solution (0.4 ml of Hach Cat. 675-49) was then added to each tube, including the control. TANNIVER® 3 reagent (0.04 ml) was added to each tube and thoroughly mixed. The tubes were then incubated at room temperature for 30 minutes. The sample was transferred to a quartz cuvette, and the absorbance read at 700 nm in the spectrophotometer. After correcting for the absorbance of the sample blank cuvette,

the absorbance of each alginate solutions was plotted against the tannic acid standard curve to determine the number of micrograms of tannic acid equivalents per milligram of sodium alginate sample. The alginate of Example 1 was found to contain 1.0 micrograms of tannic acid equivalents per milligram of sodium alginate (0.1 wt.%). The alginate of Example 2 was found to contain 0.7 micrograms of tannic acid equivalents per milligram of sodium alginate (0.07 wt.%).

For comparison, the initial alginates used to prepare the alginates of Example 1 and Example 2 were assayed for polyphenol content using the polyphenol/tannic acid assay described above. Each of the initial alginates used to prepare the purified alginates of Examples 1 and 2 was found to contain about 2.0 micrograms of tannic acid equivalents per milligram of alginate. The purification process of Example 1 provides a reduction in tannic acid equivalents greater than about 90% and the process of Example 2 provides a reduction in tannic acid equivalents greater than about 90% relative to the initial alginate compositions. The process of the present invention is effective in substantially reducing the level of polyphenols in the initial alginates.

Example 4-Fucose Sugar Analysis

The content of fucose in alginate samples can be determined by gas chromatography-mass spectrometry (GC-MS) analysis. To prepare samples for GC-MS analysis, 1-3 mg of each sample was weighed and placed in a screw top (100 mm x 13 mm) test tube. H_2SO_4 (0.5 ml at 1N) containing 50 μg inositol as an internal standard was then added to each tube. Standard tubes containing 10.0, 1.0, 0.1 and 0.01 μg fucose were then prepared in a similar manner.

All tubes were heated for 1 hour (or 3 hours) at 121°C. After heating, the tubes were cooled and a stoichiometric quantity of barium chloride was added. The neutralized tubes were centrifuged (10 min, 1500 x g) to remove the

barium sulfate produced. Water remaining in the samples was evaporated under an air stream.

The samples were then reduced with sodium borohydride (1 mg/ml NaBH_4 in 1N NH_4OH , 0.5 ml solution, 1 hour at room temperature). After reduction, excess borohydride was decomposed by addition of 200 microliters of glacial acetic acid and evaporated under an air stream.

The reduced samples were acetylated using 200 microliters of acetic anhydride and 20 microliters of 1-methylimidazole (10 min at room temperature). The samples were then partitioned between 2 ml water and 2 ml methylene chloride. The water phase was removed and the remaining organic phase was evaporated. Fifty microliters of acetone were then added to each sample and an aliquot of the sample was injected into a 5890 HP gas chromatograph coupled to a 5970 HP mass selective detector. The GC separation was performed by a J & W DB-23 column (30 meters, 0.25 mm I.D.) using a temperature gradient running from 160 to 210°C at 3°/min. MS analysis was performed by comparison of retention times and of spectral comparisons with authentic standards.

The results of fucose analysis for the initial alginates and purified alginates of Examples 1 and 2 are shown in the table below.

Table: Fucose Analysis Results

	SAMPLE	FUCOSE
		(μg fucose/mg sample)
30	Ex. 1 (initial)	2.91 (0.291 wt.%)
	Ex. 1 (purified)	<.01 (<0.001 wt.%)
	Ex. 2 (initial)	0.70 (0.07 wt.%)
	Ex. 2 (purified)	0.09 (0.009 wt.%)

The fucose level in Example 1 (purified) is reduced by a factor of more than 300 relative to the fucose level in Example 1 (initial). The fucose level in alginate Example 2 (purified) is reduced by a factor of 8 relative to Example 2 (initial).

Example 5-Preparation of Pancreatic Suspension Islet

Pancreatic islets isolated from rat were washed with isotonic saline, were suspended in an alginate solution prepared by dissolving the alginate prepared by the procedures of Example 1 and Example 2 at a concentration of 10,000 islets per ml in 1.9 wt.% purified alginate in 10 mM HEPES, 0.01 M sodium citrate, containing sufficient sodium chloride required for isoosmolarity (about 0.81 wt.%), the final solution having a viscosity of about 50 centipoises at 32°C. The islets had an approximate average diameter of 150 μ m. This procedure was repeated with dog islets.

Example 6-Coating of Pancreatic Islets

Using a DC electrostatic voltage of 8 KV provided by a van de Graaff generator between needle tip and grounded 0.117 M aqueous calcium chloride solution at ambient temperature, a suspension of pancreatic islets (25 islets per μ L) prepared by the procedure of Example 5 was passed through a 20 gauge needle at a flow rate of approximately 200 μ l/min. The suspension emerged from the needle as a thin, attenuated stream which transformed into droplets, the droplets being collected in the calcium chloride solution. The droplets were gelled by reaction with the calcium ion in the solution. The calcium alginate coatings on the islets were smooth and uniform and had an approximate thickness of about 130 μ m. The total coated particle had an average diameter of about 360 μ m.

This process was repeated with dog islets prepared by the procedure of Example 5.

Example 7-Pancreatic Islet Transplant
into Diabetic Mice (IP)

Host Balb/C mice were rendered diabetic by IP injection of streptozocin (250 mg/kg) at 50 mg/mL in 0.1 M citrate buffer, pH 4.5 several days prior to transplant.

Coated dog islets of Langerhans prepared by the procedure of Example 6 were injected IP, 2000-3000 islets per mouse, into one group of mice. The mice became and

remained euglycemic for over 72 weeks (18 months). Several mice returned to the diabetic state several weeks after implantation. These mice were sacrificed and the coated islets examined. The alginate-coated islets were found to be viable, free from fibrosis and free from macrophage overgrowth (only 2-10 macrophages per coated islet capsule).

Spheres formed from the same alginate (without cells) were injected IP into a control group of Balb/C mice. The mice were sacrificed at intervals for periods of a few days to several weeks. The alginate spheres were examined histologically and found to be free from fibrosis and substantially free from macrophage overgrowth.

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

WHAT IS CLAIMED AS NEW AND DESIRED TO BE
SECURED BY LETTERS PATENT OF THE UNITED STATES IS:

1. A non-fibrogenic alginate composition prepared by
a process, comprising

5 (a) preparing an aqueous alginate solution comprising
alignate and a divalent metal ion-chelating agent;

(b) contacting said alginate solution with bleached
activated carbon in an amount and for a time sufficient to
adsorb any fucans and other contaminants present in said
10 alginate to produce a non-fibrogenic alginate and then
removing said activated carbon from said alginate solution;

(c) adding ethanol to said alginate solution in an
amout effective to precipitate said alginate from said
alginate solution; and

15 (d) isolating said precipitated alginate.

2. The composition of Claim 1, wherein the process
further comprises

(e) coating a viable, physiologically active tissue or
cell with said precipitated alginate and gelling said
20 coated alginate by adding divalent metal ions thereto.

3. The composition of Claim 1, wherein
the fucans comprise fucose, polyphenols and protein;
the amount of fucose remaining in the composition is
less than about 0.02 wt.%; and

25 the amount of polyphenol is less than about 0.2 wt.%
in terms of tannic acid.

4. The composition of Claim 3, wherein the amount of fucose remaining in the composition is less than about 0.01 wt.%. .

5. The composition of Claim 3, wherein the amount of fucose remaining in the composition is less than about 0.005 wt.%. .

6. The composition of Claim 3, wherein the amount of polyphenol remaining in the composition is less than about 0.1 wt.% in terms of tannic acid. ,

10 7. The composition of Claim 3, wherein the amount of polyphenol remaining in the composition is less than about 0.075 wt.% in terms of tannic acid.

8. The composition of Claim 1, wherein the weight ratio mannuronate/(mannuronate + guluronate) in said alginate is about 0.1 to 0.95.

9. The composition of Claim 8, wherein said ratio is about 0.15 to 0.85.

10. The composition of Claim 8, wherein said ratio is about 0.25 to 0.75.

20 11. The composition of Claim 1, wherein the molecular weight of said alginate is about 2 to 350 kilodaltons.

12. The composition of Claim 11, wherein the molecular weight of said alginate is about 4 to 250 kilodaltons.

13. The composition of Claim 11, wherein the
5 molecular weight of said alginate is about 6 to 120 kilodaltons.

14. A non-fibrogenic divalent metal ion-alginate gel, comprising divalent metal ions and an alginate having a molecular weight of about 2 to 350 kD, a weight ratio of
10 mannuronate/(mannuronate + guluronate) of about 0.1 to 0.95, wherein the amount of fucose is less than 0.02 wt.% and the amount of polyphenol is less than 0.2 wt.% in terms of tannic acid, and wherein when a cell coated with said alginate gel is implanted into a BALB/C mouse it
15 substantially fails to induce fibrosis for at least 60 days after implantation.

15. The alginate gel of Claim 14, wherein said gel fails to substantially cause fibrosis for at least 6 months after implantation.

20 16. The alginate gel of Claim 14, wherein said gel fails to substantially cause fibrosis for at least 12 months after implantation.

17. The alginate gel of Claim 14, wherein said gel fails to substantially cause fibrosis for at least 18 months after implantation.

18. An alginate gel capsule, comprising viable,
5 physiologically active tissue coated with the composition of Claim 1.

19. An alginate gel capsule, comprising viable, physiologically active tissue coated with the composition of Claim 2.

10 20. An alginate gel capsule, comprising viable, physiologically active tissue coated with the alginate gel of Claim 14.

21. The capsule of Claim 20, wherein the physiologically active tissue comprises viable,
15 physiologically active canine or rat islets of Langerhans, wherein where an effective amount of said capsule is implanted into a diabetic mouse it allows said mouse to remain euglycemic for at least 60 days after implantation.

22. The capsule of Claim 21, which allows said
20 diabetic mouse to remain euglycemic for at least 6 months after implantation.

23. The capsule of Claim 21, which allows said diabetic mouse to remain euglycemic for at least 12 months after implantation.

24. The capsule of Claim 21, which allows said
5 diabetic mouse to remain euglycemic for at least 18 months after implantation.

25. The capsule of Claim 20, wherein the coating
has a sufficiently low permeability and a sufficiently
large thickness to protect the tissue from host
10 immunological agents after transplantation; and

has sufficient permeability and a sufficiently small
thickness to permit the diffusion of sufficient cell nutri-
ents and cell products required for cell viability through
the coating.

15 26. The capsule of Claim 20, wherein the coating has
a thickness of at least about 10 μm and less than about 200
 μm .

27. The capsule of Claim 20, wherein the tissue
comprises at least one or more cell types selected from the
20 group consisting of pancreatic islet cells, neural cells,
renal cortex cells, vascular endothelial cells, thyroid
cells, adrenal cells, thymic cells, ovarian cells and
hepatic cells.

28. The capsule of Claim 27, wherein the tissue cells are pancreatic islet cells.

29. The capsule of Claim 20, wherein the divalent metal ions comprise calcium ions.

5 30. A pharmaceutical composition, comprising the capsules of Claim 20 and a pharmaceutically acceptable carrier.

31. The pharmaceutical composition of Claim 30, wherein the tissue cells are pancreatic islet cells.

10 32. A process for coating tissue, comprising
(a) preparing an aqueous alginate solution comprising alginate and a divalent metal ion-chelating agent;

(b) contacting said alginate solution with bleached activated carbon in an amount and for a time sufficient to
15 adsorb fucans present in said alginate to produce a non-fibrogenic alginate and then removing said bleached activated carbon from said alginate solution;

(c) adding ethanol to said alginate solution in an amount effective to precipitate said alginate from said
20 alginate solution;

(d) isolating said precipitated alginate; and

(e) coating viable, physiologically active tissue with said precipitated alginate.

33. A process for producing a non-fibrogenic alginate gel, comprising
obtaining alginate and a divalent metal ion chelating agent;

5 steps (a) to (e) of the process of claim 32; and
gelling said coated alginate by adding divalent metal ions thereto.

34. The process of Claim 32, wherein said bleached activated carbon is obtained by contacting activated
10 charcoal having a particle size of about 100 mesh or finer with an about 0.005 to 0.50 M sodium hypochlorite solution for about 5 to 30 minutes.

35. The process of Claim 34, wherein the ratio of bleached activated carbon to alginate in said alginate
15 solution is about 0.5:1 to 1:10 (w:w).

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/05461

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61F 2/02; A01N 1/02; C12N 11/04

US CL : 424/422, 423, 424; 435/1, 182, 240, 241; 128/IR

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/422, 423, 424; 435/1, 182, 240, 241; 128/IR

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,663,286 (TSANG ET AL.) 05 MAY 1987, SEE COLUMN 3, LINES 4-38; COLUMN 5, LINES 51-57; COLUMN 7, LINES 20-23.	1-35
Y	US, A, 4,689,293 (GOOSEN ET AL.) 25 AUGUST 1987, COLUMN 3, LINES 33-42, 66-68; COLUMN 4, LINES 1-11.	1-35



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

12 JULY 1993

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AUG 19 1993

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